Behavior of Fecal Indicator Bacteria and Enteric Viruses in the Estuarine Environment

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論文内容要旨

1. Introduction

Gastroenteritis caused by enteric viruses is a serious concern even in developed countries including Japan. The water environment is contaminated with enteric viruses excreted in feces and vomits of gastroenteritis patients since they are not removed and inactivated completely in existing wastewater treatment processes. River and estuarine sediment is suggested to play an important role in transmission of microbes in the water environment and it is necessary to take countermeasures against gastroenteritis considering the behavior of enteric viruses in the water environment including sediment. However, although effective methods to recover bacteria from sediment are available, preparation methods for viruses, especially using molecular detection methods, are still under development. In this study, preparation methods for viruses in sediment were developed and evaluated by a qPCR method. A field survey was conducted at the Takagi River estuary and the behavior of fecal indicator bacteria and *Norovirus* (NoV) were investigated. Moreover, numerical analysis was performed to evaluate the behavior of fecal coliforms and NoV in the estuarine environment.

2. Development of a sample preparation method of virus RNA from sediment for its quantitative detection

2.1 Materials and methods

Sediment samples collected at St.G in the Takagi River estuary (Figure 1) were used for the development of a sample preparation method. There are two main types of sample preparation methods to recover virus RNA from sediment. One is an indirect preparation method, consists of elution of virus particles using some buffer solutions, centrifugation to remove sediment, concentration of the supernatant, and extraction of virus RNA from the virus concentrate. The other is a direct preparation method, consists of virus capsid lysis within sediment, extraction of virus RNA, concentration and purification of the extracted RNA. In this study, five indirect preparation methods (Method A – E) and four direct preparation methods (Method F – I) were evaluated based on recovery rates of *Poliovirus* type 1 (PV1) which was inoculated into sediment.

Table 1 and 2 outline the conditions of indirect and direct preparation methods evaluated in this study. In Method A and B, glycine-NaOH buffer (pH 9.0) was used and the supernatants were concentrated with membrane filtration methods developed by Gerba *et al.* (1977) and Katayama *et al.* (2002), respectively (Table 1). Method C was modified from Method B with one modification: TE buffer (pH 7.2) containing Laureth-12 was used to prevent elution of humic substances from sediment. In Method D and E, PEG precipitation method was applied instead of the filtration methods. Virus RNA was then extracted from the virus concentrate.

In Method F and G, virus capsid was lysed by SDS without EDTA to prevent elution of humic substances from sediment (Table 2) and virus RNA was extracted with PCI and concentrated with isopropanol. Virus RNA was purified using a DEAE-cellulose column (Ikeda, *et al.*, 2004) in Method F and magnetic beads in Method G, respectively. In Method H, TRIzol Reagent containing guanidine isothiocyanate was used and virus RNA was purified using magnetic beads. In Method I, EDTA was included to the lysis buffer.

2.2 Results and discussion

Figure 2 shows recovery rates of PV1 in Method A through I. The recovery rate (geometric mean) in Method B was the highest (5.4%) in the indirect preparation methods. As mentioned by Johnson *et al.* (1984), the low recovery rates may be because of the particle size distribution of the sediment, especially composition of silt and clay. They tested various eluents to recover PV1 from some kinds of sediment and mean recovery rates were 3.2% and 0.9% from the sediment containing 4.6% and 17.4% clay, respectively. Those recovery rates were comparable to this study since the sediment at St.G was mainly consisted of silt (67%) and clay (13%). In Method D, humic substances were co-concentrated with PEG and PV1 was not detected due to the inhibition of RNA extraction and RT-qPCR. Other eluents were also tested; however, PV1 recovery rates were at most 1%. This was because viruses were not dispatched from sediment particles efficiently or humic substances were co-eluted and

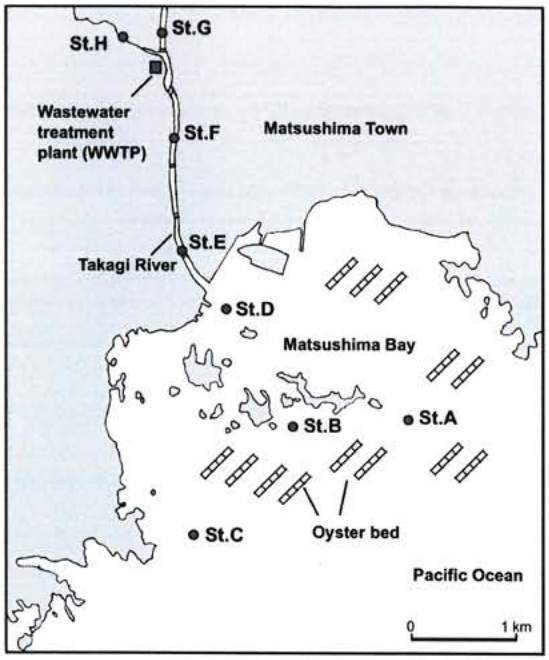

Figure 1. Locations of sample sites.

Table 1. Condisions of indirect preparation methods

Method	Elution buffer	Concentration method
A	Glycine-NaOH	Membrane filtration method by Gerba *et al.* (1977)
B	Glycine-NaOH	Membrane filtration method by Katayama *et al.* (2002)
C	TE-Laureth-12	Membrane filtration method by Katayama *et al.* (2002)
D	Glycine-NaOH	PEG precipitation method
E	TE-Laureth-12	PEG precipitation method

Table 2. Condisions of direct preparation methods

Method	Lysis buffer	Purification method
F	SDS without EDTA	DEAE-cellulose
G	SDS without EDTA	Magnetic beads
H	TRIzol Reagent	Magnetic beads
I	SDS with EDTA	Magnetic beads

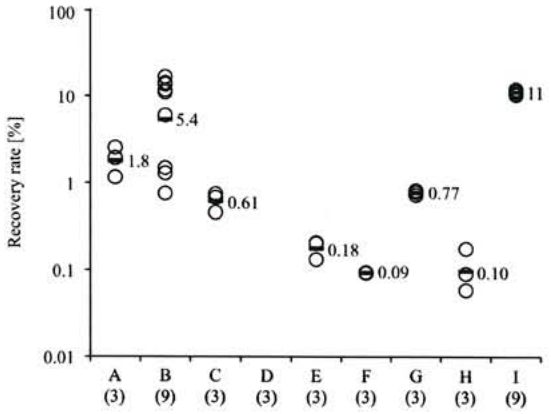

Figure 2. Recovery rates of PV1 in Method A through I. Values beside the plots mean geometric mean of the recovery rate. Values in parentheses mean the number of samples.

co-concentrated. These results suggested that it was quite difficult to separate virus particles from humic substances because humic substances have wide range of molecular weight and isoelectric point.

As for the direct preparation methods (F – I), the recovery rate in Method I was the highest (11%, Figure 2). In Method F and G, the lysis buffer without EDTA reduced the elution of humic substances; however, it also reduced the recovery of PV1 RNA. In Method F, the recovery rate of PV1 RNA in the purification step using the DEAE-cellulose column was low and the eluted humic substances were not completely separated from PV1 RNA even after an additional isopropanol precipitation. On the other hand in Method G thorough I, the recovery rates in the purification step using magnetic beads were higher and it was found that magnetic beads which can specifically capture the target RNA was effective to purify RNA from the humic substances.

3. Field survey on the behavior of fecal indicator bacteria and enteric viruses in the Takagi River estuary

3.1 Materials and methods

Field surveys were conducted at the Takagi River estuary monthly from Nov. 2007 to Apr. 2009. The sediment samples (N = 108) were collected at St.A through E, and G (Figure 1). The sample obtained by an Ekman-Birge type bottom sampler covered a square area of 15 by 15 cm and the top layer of 1 cm was collected. The water samples (N = 180) were collected at all sample sites including influent and effluent of the wastewater treatment plant (WWTP). The sediment and water samples were tested for fecal coliforms, *Bacteroides* spp., and NoV. Virus RNA in sediment was prepared by the developed direct preparation method (Method I).

3.2 Results and discussion

Figure 3 shows geometric mean (GM) concentrations of fecal coliforms in the sediment and water samples. In all sample sites, the concentration in sediment was 12 to 663 times higher than those in water. The concentration in the river sediment at St.G was the highest (GM = 7.5 x 10^4 CFU/100 g dry weight), followed by estuarine sediments and then marine sediments. This may be because fecal coliforms cannot survive for a long period of time in seawater. That was why the differences of the concentration in sediment and water at the bay were larger than those at the river (Figure 3).

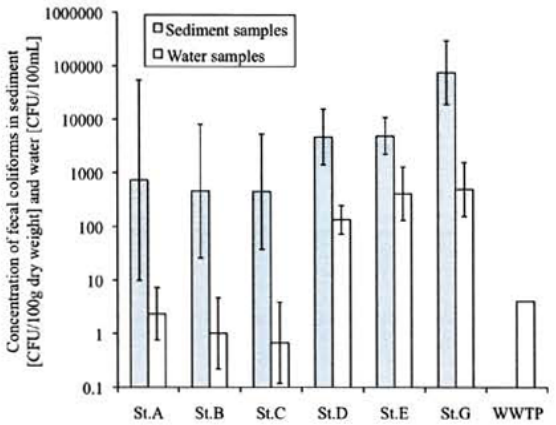

Figure 3. Concentrations of fecal coliforms in the sediment and water samples.

Human-specific *Bacteroides* spp. was detected and the sediment in the study area proved to be contaminated with bacteria of human-fecal origin. This study is the first to apply host-specific *Bacteroides* spp. for fecal source tracking in sediment.

Figure 4 shows concentrations of NoV in the water samples. Concentrations (GM) of NoV in the Takagi River and the Matsushima bay were 9.9 copies/L (N = 37) and 0.73 copies/L (N =

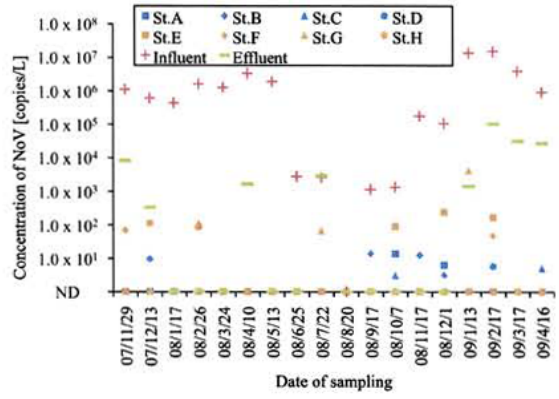

Figure 4. Concentrations of NoV in the water samples.

40), respectively in winter season. In Sep. 2008, NoV was detected from seawater (14 copies /L) even though they were not from effluent of WWTP and river waters. This was partly because NoV would be resuspended to the seawater from sediment at the river mouth or the bay. NoV was detected from the sediment samples collected at St.A in Mar. 2008, although the concentration was below the quantification limit. This is the first report of detection of NoV in sediment; however, it was recommended that more effective preparation methods for enteric viruses in sediment be developed for their direct monitoring.

4. Evaluation of the behavior of NoV and fecal coliforms in the Takagi River estuary based on numerical analysis

4.1 Materials and methods

Behavior of NoV and fecal coliforms was numerically analyzed using a hydraulic model composed of tidal flow and diffusion of solutes. The Takagi River estuary (7.5 x 7.5 km^2) was divided into 150 x 150 grid cells. Concentrations of microbes (13 copies/L for NoV and 495 CFU/100 mL for fecal coliforms at upstream, 0 copies/L and 0 CFU/100 mL in the Pacific Ocean) were given as boundary conditions based on observed data in the field survey.

4.2 Results and discussion

Figure 5 shows estimated concentration of NoV in the Takagi River estuary at ebb tide. The concentration at a coastal area was approximately 1 log higher than those at St.A through C. If oysters accumulate NoV constantly, the risk of gastroenteritis caused by NoV in oysters from the coastal area would be 1 log higher.

Figure 6 shows estimated and observed concentrations of NoV. The estimated concentration was well matched to the observed concentration in the river. However, the estimated concentration at the river mouth (St.D) was higher than the observed concentration. And on the other hand, the estimated at the bay was lower than the observed. This result suggested that behavior of NoV in the river was similar to that of dissolved organic matters. With the addition of sedimentation of NoV at the river mouth and resuspension at the bay, the model would be more reliable to simulate the behavior of NoV in the estuarine environment.

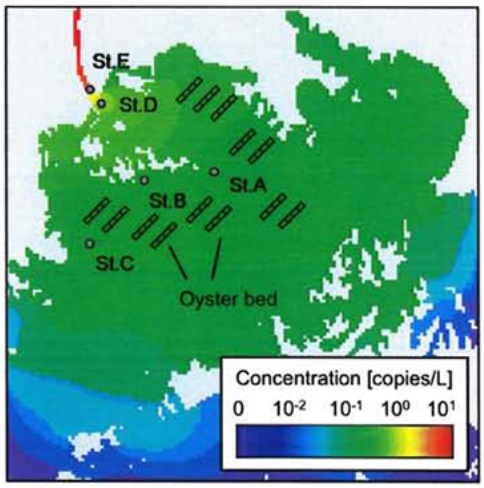

Figure 5. Estimated concentration of NoV in the Takagi River estuary at ebb tide.

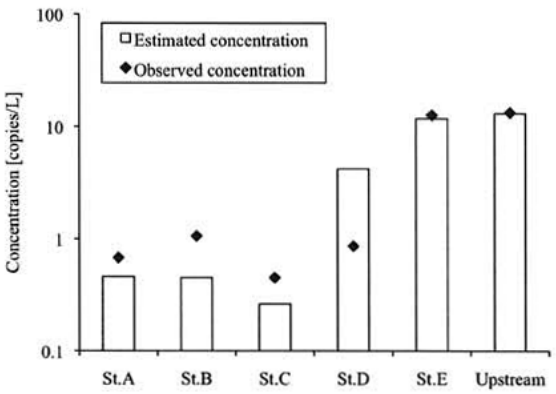

Figure 6. Estimated and observed concentrations of NoV at St.A through E and upstream of the Takagi River.

References

Gerba, *et al.* (1977) Appl Environ Microbiol, 34, 158-163.

Ikeda, *et al.* (2004) Microbes Environ, 19, 301-309.

Johnson, *et al.* (1984) Appl Environ Microbiol, 48, 581-585.

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論文審査結果の要旨

本論文では，河口沿岸域の底泥を含めた水環境中における糞便汚染指標細菌と腸管系ウイルスの挙動の解明に向けて，底泥からの新規なウイルス検出手法を開発した。そして，その手法を用いた現地調査と潮流・拡散モデルを用いた数値解析により，河口沿岸域における微生物の挙動を評価し，重要で新たな多くの知見を得た。

第１章では，研究の背景として，腸管系ウイルスを原因とする感染性胃腸炎の発生と感染者の糞便を介して水環境中に排出されるウイルスの挙動について既往の知見を整理している。具体的には，感染性胃腸炎の季節的流行メカニズムの解明やその対策には水環境中における腸管系ウイルスの動態を解明する必要があること，河口沿岸域の底泥は腸管系ウイルスを含めた病原微生物のリザーバーとして機能する可能性があり底泥を含めた議論が重要であること，底泥からウイルスを検出する手法がまだ確立されていないことが課題の１つであることが述べられている。

第２章では，水環境中の糞便汚染指標細菌および腸管系ウイルスについて，既往の知見をさらに詳しくまとめている。腸管系ウイルスとそれらが引き起こす胃腸炎の特徴，水試料および底泥試料からの糞便汚染指標細菌および腸管系ウイルスの検出方法，水中および底泥中における糞便汚染指標細菌と腸管系ウイルスの生残性などについて示された。

第３章では，底泥試料からウイルス粒子を回収・濃縮し，ウイルス濃縮液から遺伝子（RNA）を抽出する，ウイルス RNA の間接回収法を開発した。様々な溶出バッファーや濃縮法を検討し，既往の手法を用いた場合よりもおよそ１オーダー高いウイルス回収率を実現させた。しかし，この手法では，研究対象地域の底泥試料に対してこれ以上の回収率の向上が難しいこと，環境試料からのウイルス検出には試料の濃縮の面で限界があることが明らかにされた。

第４章では，底泥試料からウイルス RNA を直接抽出し，その後の濃縮・精製手法を工夫することで，回収率と濃縮倍率の向上を実現させたウイルス RNA の直接回収法を開発した。これまでウイルス RNA の濃縮・精製に適用された例がない磁気ビーズを使用したことで，第３章で開発した手法よりもウイルス回収率をさらに２倍向上させ，濃縮倍率も 50 倍高めることができた。

第５章では，研究対象地域の高城川および松島湾で 18 ヶ月間に渡って採取した海水，河川水，下水，下水処理水，および底泥試料から糞便汚染指標細菌である糞便性大腸菌群とバクテロイデス属細菌，腸管系ウイルスとしてノロウイルスを定量検出し，河口沿岸域におけるそれらの挙動を明らかにした。同時に，日本の河口沿岸域底泥中における糞便性大腸菌群濃度を初めて報告した。また，これまでに環境水の糞便汚染起源解析に用いられてきたバクテロイデス属細菌を底泥から検出したことで，底泥の糞便汚染起源解析にも適用可能であることを明らかにした。さらに，第４章で開発した手法を用いて，初めて河口沿岸域の底泥からノロウイルス遺伝子を検出し，ノロウイルスの水環境中における挙動について重要で新たな知見を示した。

第６章では，第５章の実測データにもとづき，高城川流域における微生物の挙動を数値解析し，河川流下中や河口域での微生物の挙動を明確に示すことが出来た。

第７章は，結論である。

以上のことより，本論文は，河口沿岸域の底泥から効率的に腸管系ウイルスを検出できる新規な手法を開発し，環境試料からノロウイルス遺伝子を検出することを可能とした。また，現地調査と数値解析の両面から河口沿岸域における糞便汚染指標細菌と腸管系ウイルスの挙動を明らかにした。従って，その成果は環境工学の発展に寄与するところが少なくない。

よって，本論文は博士(工学)の学位論文として合格と認める。